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(54) Title: A FUNGUS WHEREIN THE areA GENE HAS BEEN MODIFIED AND AN areA GENE FROM ASPERGILLUS ORYZAE

(57) Abstract

The present invention relates to fungi, which do not produce proteases. The fungi of the invention are useful as hosts for the production of proteins susceptible of proteolytic degradation by the proteases usually produced, and the invention consequently encompasses processes for the production of proteins of interest in high yields by using the fungi of the invention. The invention also comprises methods for producing such fungi and DNA constructs to be used in these methods.

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A fungus wherein the areA gene has been modified and an areA gene from Aspergillus oryzae.

FIELD OF THE INVENTION

The present invention relates to fungi, which do not produce proteases. The fungi of the invention are useful as hosts for the production of proteins susceptible to proteolytic degradation by the proteases usually produced, and the invention consequently encompasses processes for the production of proteins of interest in high yields by using the fungi of the invention. The invention also comprises methods for producing such fungi and DNA constructs to be used in these methods.

BACKGROUND OF THE INVENTION

Fungi, and especially filamentous fungi, are widely used commercially because of their ability to secrete remarkably high levels of proteins

Among the filamentous fungi species belonging to the genus *Aspergillus* have a long history of commercial use for the production of endogenous and lately also heterologous proteins.

One disadvantage with most microorganisms used for the production of proteins is the inherent production of proteases which may subject a protein product of interest to degradation due to proteolysis.

Various ways of avoiding this have been envisaged. Among other solutions it has been suggested to delete or disrupt the genes encoding the various proteases. Unfortunately the fungi produce a high number of proteases making such a solution more or less unrealistic.

A need is therefore persisting for strains of filamentous fungi exhibiting no or very low levels of protease production.

For a number of years it has been known that the regulatory gene *areA* which mediates nitrogen metabolite repression in *A. nidulans* influences the production of extracellular proteases (Arst & Cove, *molec. gen. Genet.* **126**, (1973) 111-141).

The *areA* gene from *A. nidulans* has been cloned (Caddick *et al.*, *EMBO Journal* **5**, (1986) 1087-1090) and various modifications made to it to evaluate functions of different regions in the activator protein encoded by this gene (Stankovitch *et al. Mol. Microbiol.* **7**, (1993) 81-87). Furthermore the gene coding the corresponding function in *A. fumigatus* apparently has been cloned recently (Hensel *et al.* 2nd European Conference on Fungal Genetics, April 28 to May 1, 1994, Book of Abstracts, E11).

From the literature a single use is also known of a strain of *A. nidulans* of genotype *argB areA1* as a host for the production of t-PA (Upshall *et al. Biotechnology* **5**, (1987) 1301-1304). In this example only the *argB* genotype is used as a selection marker through its arginine prototrophy, while the *areA* genotype is simply a coincidence.

The present invention has as an object the alleviation of the need for protease free filamentous fungi.

SUMMARY OF THE INVENTION

The present invention consequently relates to fungi, wherein the *areA* gene by recombinant DNA technology has been modified such that it cannot be expressed in a way providing for a functional AreA activator.

The invention furthermore relates to methods for producing such fungi, obtained by deletion of the *areA* gene.

This may be obtained through a method comprising

i) cloning of the *areA* gene from a fungus of interest,

ii) producing a DNA construct comprising the *areA* gene wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the construct, and

iv) selecting transformants which are *areA*$^-$.

The information obtained from the above mentioned cloning of the *areA* gene may also be used in connection with the well-known anti-sense technology, to construct an expression plasmid giving rise to synthesis of a RNA molecule complementary to the mRNA transcribed from the *areA* gene, and to transform the fungus of interest therewith.

The invention furthermore relates to DNA constructs intended for use in the above mentioned methods.

Furthermore the invention relates to methods of producing a desired protein or gene product, especially secreted proteins, whereby a fungal host modified and optionally transformed with a DNA construct comprising at least a DNA sequence coding for the protein or gene product of interest, is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

When working with the invention it was surprisingly found that the fungi of the invention produces such secreted proteins in a much improved yield.

It was also surprisingly found that the only nitrogen source capable of providing good growth of the *A. oryzae areA*$^-$ strains was glutamine.

Lastly the invention relates to protein products produced by the above methods.

BRIEF DESCRIPTION OF THE DRAWING

The invention is described in further detail in the following parts of the specification with reference to the Examples and the drawing, wherein

Fig.1 shows the steps involved in the construction of HowB101,

Fig. 2 shows the steps involved in the construction of pSK5 and pSK9,

Figs. 3a and 3b show the steps involved in the construction of pToC266,

Fig. 4 shows the steps involved in the construction of pMT1606,

and

Fig. 5 shows the steps involved in the construction of pToC56.

DEFINITIONS

In the present specification the following definitions are used

The expression *areA*Δ means a strain in which the *areA* gene is deleted.

The expression *areA*$^-$ means a strain which does not produce a functional AreA activator. The term "loss of function" is also often used for this.

The expression "anti-sense technology" describes methods such as disclosed in US Patent No. 5,190,931.

DETAILED DESCRIPTION OF THE INVENTION

As indicated the present invention relates in its first aspect to fungi, wherein the *areA* gene by recombinant DNA technology has been modified such that it cannot be expressed in a way providing for a functional AreA activator.

This object may specifically be obtained by deletion or disruption of the *areA* gene.

The cloning of the *areA* gene is described in the Examples.

AreA homologs from other fungi could be cloned either by cross hybridization with one of the already known genes or by complementation of *areA* mutants; e.g. *A. nidulans areA*-18 or the *A. oryzae areA* deleted strain described in this application.

Methods for deleting or disrupting a gene are specifically described in WO 90/00192 (Genencor).

Methods for substituting DNA in a gene are also generally known, and can be accomplished by substituting one or more continuous parts of the gene, but it may also be obtained by site directed mutagenesis generating a DNA sequence encoding a AreA activator variant that is not functional.

Another method by which such an object may be obtained is by using anti-sense technology.

The anti-sense technology and how to employ it is described in detail in the aforementioned US Patent No. 5,190,931 (University of New York).

A further method of obtaining said inactivation is by inserting extra DNA internally in the *areA* gene, thereby giving rise to the expression of a dysfunctional activator protein.

In connection with this method information provided by the cloning can be used to make DNA constructs that can be integrated into the *areA* gene, and even replace it with another gene, such as the *pyrG* gene.

A further method of avoiding the presence of the *areA* activator is by interfering with the regulation of the expression signals regulating the expression of the *areA* gene itself.

According to the invention the fungus preferably belongs to a genus selected from the group comprising *Aspergillus*, *Trichoderma*, *Humicola*, *Candida*, *Acremonium*, *Fusarium*, and *Penicillium*

Among these genera species selected from the group comprising *A. oryzae*, *A. niger*, *A. awamori*, *A. phoenicis*, *A. japonicus*, *A. foetidus*, *A. nidulans*, *T. reesei*, *T. harzianum*, *H. insulens*, *H. lanuginosa*, *F. graminearum*, *F. solani*, *P. chrysogenum*, and others are preferred.

As indicated the invention also is meant to encompass the method for producing the fungi of the first aspect of the invention, and wherein said inactivation has been obtained by deletion of the *areA* gene, which method comprises

i) cloning of homologues of the *areA* gene from a fungus of interest,

ii) producing a DNA construct comprising the *areA* gene wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the construct, and

iv) selecting transformants which are *areA*⁻ .

Also included is the method for producing the fungi, wherein the inactivation has been obtained by using anti-sense technology. Such a method comprising

i) construction of an expression plasmid which gives rise to synthesis of a RNA molecule complementary to the mRNA transcribed from the *areA* gene,

ii) transformation of the host fungus with said expression plasmid and a suitable marker, either on separate plasmids or on the same plasmid,

iii) selection of transformants using said marker, and

iv) screening transformants for strains exhibiting a reduction in the synthesis of the AreA product, e.g. by analysis of the growth on various nitrogen sources.

A further aspect of the invention is meant to comprise DNA constructs for use in the above mentioned methods.

In respect of the former method said DNA constructs may comprise the *areA* gene wherein an internal part has been substituted, deleted, or extra DNA has been inserted.

The DNA construct may furthermore also comprise DNA sequences encoding a protein product of interest, such as those mentioned later.

In respect of the latter anti-sense method the DNA construct may comprise an inverted DNA sequence of the *areA* gene connected to a functional promoter, whereby the mRNA is at least partially complementary to mRNA produced from the *areA* gene.

A further aspect of the invention relates to a process for the production of a desired gene product, preferably a secreted gene product, whereby a fungus according to the invention is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

In the case of a gene product expressed by a heterologous gene the DNA sequence coding for the desired gene product may be a part of the DNA construct used for producing said fungus.

Normally, however, a separate transformation of the fungus of the invention is performed in order to make the fungus capable of producing the desired product.

Methods for transforming fungi are well known in the art, cf. e.g. EP 0 184 438 A2 (Gist-Brocades N.V.) and EP application no. 87103806 (Novo Nordisk A/S) and.

For indigenous products this is of course not necessary, but in order to increase the production it may be an advantage to provide for multiple copies of the gene encoding the protein of interest to be incorporated into the host.

The desired gene product is generally a peptide or protein, preferably an enzyme.

Among enzymes it is preferably selected from the group comprising proteases, such as trypsin and chymosin; lipases, cutinases, cellulases, xylanases, laccases, pectinases, etc.

Another type of desired gene product is generally a therapeutically active peptide or protein.

Among the therapeutically active peptide or protein the protein preferably is selected from the group comprising insulin, growth hormone, glucagon, somatostatin, interferons, PDGF, factor VII, factor VIII, urokinase, t-PA, CSF, lactoferrin, TPO etc.

The invention is explained in further detail in the Examples given below. These should, however, not in any way be construed as limiting the scope of the invention as defined in the appended claims.

EXAMPLES

Materials and Methods

Strains

A. *oryzae*, IFO4177: available from Institute for Fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-Ku, Osaka, Japan.

ToC913: The construction of this strain is described in the Examples.

Genes

areA: This gene codes for a regulatory protein controlling nitrogen catabolism.

pyrG: This gene codes for orotidine-5'-phosphate decarboxylase, an enzyme involved in the biosynthesis of uridine.

bar: This gene was originally isolated from *Streptomyces hygroscopicus* and codes for phosphinothricin acetyltransferase. The enzyme modifies phosphinothricin (=glufosinate) and thereby inactivates this compound which is toxic to bacteria, fungi and plants.

Plasmids

pUC118: Viera and Mesing *J. Meth. Enzymol.* 1987 153 3-11

pSO2: The construction of this plasmid is described in the Examples.

pJers4: A 2.0 kb subclone of pSO2 in pUC118. pJers4 contains a functional *A. oryzae pyrG* gene.

pSO5: The construction of this plasmid from pSO2 is described in the Examples.

pToC56: The construction of this plasmid is described in EP application no. 87103806.

pToC266: The construction of this plasmid is described in the Examples.

pMT1606: The construction of this plasmid from pBP1T (B. Straubinger *et al*. Fungal Genetics Newsletter **39**(1992):82-83) and p775 (EP application no. 87103806) is described in the Examples.

p777: The construction of this plasmid is described in EP application no. 87103806.

pHW470: The construction of this plasmid is described in the Examples.

EXAMPLE 1

Construction of an *Aspergillus oryzae areA*Δ strain.

The *areA*Δ strain was constructed by the following steps. The *A. oryzae pyrG* gene was cloned and an *A. oryzae pyrG* mutant strain was isolated. The *areA* gene from *A. oryzae* was cloned. The *pyrG* mutant was transformed with a plasmid carrying the *pyrG* gene inserted between DNA fragments upstream and downstream from the *areA* gene. The coding region for *areA* was not present on the plasmid. Transformants were selected for their ability to grow in the absence of uridine and in the presence of chlorate. This double selection selects both for a functional *pyrG* gene and for *areA* minus. Strains obtained by this selection procedure were finally screened by Southern analysis to identify those in which the chromosomal *areA* gene was substituted by the *pyrG* gene.

<u>Cloning of the *A. oryzae pyrG* gene.</u>

The *A. oryzae pyrG* gene was cloned by cross hybridization with the *A. niger pyrG* gene (W. van Hartingsveldt et al., Mol. Gen.

Genet 206:71-75 (1987)). A lambda library of partial *SauIIIA* digested *A. oryzae* IFO4177 DNA was probed at low stringency with a 1 kb DNA fragment from the *A. niger pyrG* gene. A 3.8 kb *HindIII* fragment from a positive clone was subcloned into a pUC118 vector. The resultant plasmid, pSO2, was shown to contain the *pyrG* gene by complementation of an *A. niger pyrG* mutant.

<u>Construction of an *A. oryzae pyrG* minus strain.</u>
A *pyrG* deletion plasmid, pSO5, containing about 1 kb of *pyrG* flanking sequences on each end was constructed from the plasmid pSO2. *A. oryzae* IFO4177 was transformed with this construct and transformants were selected by resistance to 5-fluoro-orotic acid, a phenotype characteristic of *pyrG* mutants. One transformant, HowB101, was shown by Southern analysis to have the expected deletion at the *pyrG* locus. Being a *pyrG* mutant HowB101 requires uridine for growth. HowB101 can be transformed with the *wt pyrG* gene by selection for ability to grow without uridine.

The steps involved in the construction of HowB101 are illustrated in Fig. 1.

<u>Cloning of the *areA* gene.</u>
The *A. oryzae areA* gene was cloned by cross hybridization to the *A. nidulans areA* gene (B. Kudla et al., EMBO J. 9:1355-1364 (1990)). A genomic library of *A. oryzae* IFO4177 was prepared by partial digestion of chromosomal DNA with *SauIIIA* and cloning of the obtained DNA fragments into the vector λGEM-II (obtained from Promega). Cross hybridization of the library with the *A. nidulans areA* gene was performed in 40% formamide at 37°C. Hybridizing λ clones were isolated and from these fragments were sub-cloned into the vector pBluescript SK+ (obtained from Stratagene) giving rise to the plasmids pSK5 and pSK9 illustrated in Fig. 2. The cloned gene was able to complement an *A. nidulans areA* mutant, proving that it is indeed the *A. oryzae areA* homolog. 5643bp of the clone was sequenced, and comparison of the sequences of the *A. oryzae* and the *A. nidulans areA*

genes shows that they are highly homologous. The sequence of the *A. oryzae areA* gene is shown in SEQ ID No. 1.

Construction of the *areA* deletion plasmid.

In order to delete the *areA* gene from the *A. oryzae* chromosome the plasmid pToC266 was constructed. pToC266 contains a 2.1 kb DNA fragment originating upstream of the *areA* gene (isolated from pSK5) and a 1.4 kb DNA fragment originating downstream from the *areA* gene (isolated from pSK9). The two fragments are separated by appr. 3.2 kb in the genome, the coding region is situated in this part of the gene. The *A. oryzae pyrG* gene from pJers4 was inserted between the *areA* upstream and downstream DNA fragments. The construction of pToC266 is illustrated in Figs. 3a and 3b. pToC266 has a unique *EcoRI* site and was linearized by cutting with this restriction enzyme before used in transformations.

Selection of *A. oryzae areA*Δ strains.

A. oryzae HowB101 was transformed with linearized pToC266. Transformants were selected on minimal plates (Cove *Biochem. biophy. Acta* (1966) **113** 51-56) containing 5% sodium chlorate and 0.5 mM ammonium sulfate and 1% glucose. Transformants were thus subject to a double selection, both for having obtained the *pyrG* gene by being able to grow without addition of uridine and for chlorate resistance. Chlorate resistance is one of the phenotypes of *A. nidulans areA* mutants (H. N. Arst and D. J. Cove, MGG 126 : 111-141 (1973)). Weakly growing transformants were reisolated twice on the same type of plates. Three independent transformants named ToC913, ToC919 and ToC920 were subjected to growth test on different nitrogen sources. They grew well on glutamine, but weakly on other nitrogen sources tested, including ammonia. Southern analysis showed that the three strains have the lost the *areA* structural gene, which had been replaced by the *pyrG* gene.

*areA*Δ strains can also be obtained by selection of transformants of linearized pToC266 on minimal plates containing glu-

tamine as nitrogen source. In one such experiment one out of 25 transformants was an *areAΔ* strain.

EXAMPLE 2

Construction of pMT1606

A plasmid containing the *bar* gene from *Streptomyces hygroscopius* (C. J. Thompson et. al, EMBO J. 6 : 2519-2523 (1987)) inserted after the *A. oryzae* TAKA-amylase promoter and followed by a fragment containing the transcriptional terminator and polyadenylation signal from the *A. niger gla* gene was constructed.

The plasmid, pMT1606, can be used for selection of glufosinate resistant transformants of *A. oryzae*. pMT1606 was constructed by isolating the *bar* gene from the plasmid pBP1T (B. Straubinger et. al, Fungal Genetics Newsletter 39 : 82-83 (1992)) and cloning it into the fungal expression plasmid p775 described in EP application no. 87103806. Fig. 4 illustrates the construction of pMT1606.

EXAMPLE 3

Production of chymosin in ToC913 (*A. oryzae* IFO4177 *areAΔ*)

The *A. oryzae areAΔ* strain ToC913 was transformed with the plasmid pToC56 (Fig. 5), which is a fungal expression plasmid for the mammalian enzyme chymosin, by co-transformation with pMT1606. Construction of the plasmid pToC56 is described in EP application no. 87103806.

Transformants were selected for growth on minimal medium containing 10 mM ammonium and 1 mg/ml glufosinate and screened for the presence of pToC56 by the ability to produce chymosin. Three transformants were grown in shake flasks in minimal medium containing maltodextrin and glutamine for 4 days at 30°C.

Two transformants of pToC56 in IFO4177 (obtained as described in EP 87103806) as well as untransformed IFO4177 and ToC913 were grown along with the ToC913 transformants.

Samples of the fermentation broth were taken every day and applied to SDS-Page and Western blotting. The blotting membrane was incubated with chymosin specific rabbit antibody followed by goat rabbit antibody coupled to peroxidase. Staining of the membrane showed that the supernatants from transformants of IFO4177 contained small amounts of chymosin or degradation products thereof on the first and second day of fermentation and nothing later in fermentation.

Transformants of ToC913 contained at least ten times more full size chymosin. The amount of chymosin in the supernatants increased for the first two-three days and then remained constant.

Supernatants from the third and fourth day of fermentation of IFO4177, ToC913, a transformant of pToC56 in ToC913, and a transformant in IFO4177 were applied to an isoelectric focussing gel and electrophoresis was performed. The pH gradient was from 3.5 to 9.5. After electrophoresis the gel was rinsed with a buffer at pH = 7.0 containing 2 mM Zn^{2+} and overlayed with an agar containing 0.5% casein. The gel was incubated at $45^{0}C$ untill protease activity was visible.

In samples from IFO4177 three bands with protease activity could be seen; one with an alkaline pI and two with acidic pI's.

In samples from the pToC56 transformant of IFO4177 a faint reaction from chymosin could be seen, which partially overlapped with one of the acidic bands found in untransformed IFO4177, the protease with most acidic pI was barely visible, while the protease with the alkaline pI was clearly visible along with one or more band with an almost neutral pI.

In the samples from ToC913 no protease activity was detected, while the sample from the pToC56 transformant of ToC913 showed a strong chymosin signal. No other proteases were detected in samples from this transformant.

EXAMPLE 4

Production of human trypsin I in ToC913 (*A. oryzae* IFO4177 *areA*Δ)

A cDNA encoding human pancreatic trypsinogen I (TRYI) was isolated using standard procedures and the sequence published by M. Emi *et al*, *Gene* (1986) 41 : 305-310(cf. Danish patent application no. 693/95). A *BamHl* site (GGATCC) was introduced immediately upstream of the start codon (ATG(Met)) with the short sequence ACC between.

This *BamHl* site was used to fuse the cDNA to the *BamHl* linker in the Taka-amylase promoter in the fungal expression plasmid p777 described in EP application no. 87103806. The 3'end of the cDNA was fused 41 bp downstream of the stop codon to a *Nrul* site in p777. This inserts the TRYI cDNA between the *A. oryzae* Taka-amylase promoter and the *A. niger* glucoamylase transcription terminator. The resulting plasmid was called pHW470 (cf. Danish patent application no. 693/95).

pHW470 was transformed into ToC913 by co-transformation with the plasmid pMT1606. BASTA resistant transformants were reisolated twice through conidiospores. 8 transformants were grown for four days at 30°C in YPM (YPD(Sherman, F. *et al* (1981)Methods in Yeast Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY)) in which the glucose was replaced with 2% maltose). Supernatants were analysed for the content of human trypsin by SDS-PAGE followed by Western blotting and incubation with a rabbit antibody raised against porcine trypsin. The blotting membrane was then incubated with goat anti rabbit antibody coupled to peroxidase and reacted with

3-amino-9-ethyl carbazole. Supernatants from three of the transformants contained a stained band of the expected size. The concentration of trypsin in the three positive supernatants was 2-5 mg/I.

The presence of trypsin was further verified by incubation of samples of supernatants with L-Benzoyl-arginoyl-paranitro anilide (L-BAPNA). Samples from the three immuno positive strains cleaved the substrate, which resulted in the development of a yellow colour. Samples from ToC913 and IF04177 did not show any activity against this substrate. The specific activity of human trypsin in this assay in not known, it is thus not possible to calculate the concentration of trypsin in the supernatants from these data.

Transformants of pHW470 in the wild type strain IF04177 were also made. More than 20 L-BAPNA positive transformants were looked at, but it was not possible to detect any immonoreactive bands in supernatants from these transformants. The detection limit was approximately 0.5 mg/l in this assay.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S

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(ii) TITLE OF INVENTION: Novel Microorganisms

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5643 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Aspergillus oryzae

(B) STRAIN: IFO4177

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 2701..2769

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: join(2282..2700, 2770..4949)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GGTTACATGC GAAGGATTCT GATTATATTC ATGATTATGT CAAGCCTTTT CTCTCGTGTG     660
AAGAGGAGCA GAGAATCCGT ACGGGTTTAA TTTAATTTAG CGCCCTGCAG CTTCGAGAAC     720
ATCCCCAGCA ACGTTAAAAA CCACGAGCTA AAATGGGTCG CCACCGGAAG CACTCGAGTC     780
GAGAGATCGG TCGGCTCAGT ATTCGTAATA CCTGCGTTCC AGACGGTTTT GGTCGTTGGT     840
TTCACTCAGG GAACTTAATT CCAGCGGGAC CCAATATAAT TTGAATGATT CATGATACAT     900
CCATTCGTTT GAACCGATCC TGCAAGAGTT CTGTCTGATT TGGTCAACAT AGTTTTCCTC     960
TGGGGGAGAC TGGGGAAGAG TCAACACAAT GGTCAGGGAG AGAAGAATGA AAGCTCTCGC    1020
AAGTGGATGA TCATGCTACG TACTGTAGGA ATAAAATTAA TTAATGCGAG GCTGCAAGTA    1080
TCCCTGCGCC GATTTTCTCT TCTTACGGCG GGAACCAAAA AATGTGACGC TGTGATTTTC    1140
TGGAAAAGGT AAGGATGTTT AGTTTCCCAG GATTATTACT GGTTCCGTAT GTGTATGTGT    1200
ATGGATATCA TTCCGTATGG ATACGCCCGT TTCCTCCGCC CAGAACCAGT CCGTCATCCA    1260
TCCTCCACTC TTTCTTCTCT TAGAGCCTTT CCACCTCTCT TCACTTTCTT TTTCTTTCCC    1320
CCCTCCCTCT TTGCTTTCCC TCTCCCAGTA TTATTCTTAT ATTATCGGTT TGACCGTCGC    1380
CTCAGTATCG GCCCCCCGTG AATCACTTTT CGTTTCTCTT GTATTTTACT TTCCTATCTG    1440
GGATTGCTCC TCGATTAGCA GCTCTACTTC ATTCGGCCAT GTGCGTCTAG AGGGTCTAGC    1500
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AACTGACTTG GACCCCCTTC AGATCCTAGC TTCCCGATTC TTTTCCACCA CTGCTGCATC    1980
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ATTGCTTGCT GGGCTGGTTC TTGAACCTCT TCAATCGTCC TTATCTCTTT CCCCCCATCC   2160
GGCCTGTGAT TCCTATCTTT CCTTTTTTTC TTCCCTTTCT TGTTTGATCC CCCCTCCTCC   2220
CCGTCTTATC GCCTACTATC GTGATCCCCG CCCTTCCCAA TAAAGAGTAG GGCGTGTGAA   2280

C ATG TCC GGG TTA ACC CTC GGG CGA GGC CCT GGG GGC GTG CGA CCG        2326
  Met Ser Gly Leu Thr Leu Gly Arg Gly Pro Gly Gly Val Arg Pro
  1               5                   10                  15

ACT CAA ACC GCA ACT TTT ACC ACC CAC CAC CCG TCC GCC GAT GCT GAC     2374
Thr Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp
                20                  25                  30

CGC TCC TCC AAC AAC CTC CCC CCT ACC TCC TCG CAG CTG TCC GAT GAC     2422
Arg Ser Ser Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp
            35                  40                  45

TTT TCT TTC GGT TCC CCT CTG AGC CCC GCC GAC TCA CAG GCC CAT GAC     2470
Phe Ser Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp
        50                  55                  60

GGC CTA CTT CAG GAC TCC CTC TTC CCT GAA TGG GGG TCT GGT GCG CCT     2518
Gly Leu Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro
    65                  70                  75

CGA CCC GGC ATT GAC AGT CCG GAT GAG ATG CAG AGG CAA GAT CCG CTA     2566
Arg Pro Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu
80                  85                  90                  95

GCG ACT CAA ATA TGG AAG CTC TAT TCT AGG ACC AAG GCC CAG TTG CCC     2614
Ala Thr Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro
                100                 105                 110

AAC CAG GAG CGT ATG GAA AAC CTG ACC TGG CGG ATG ATG GCG ATG AGT     2662
Asn Gln Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser
            115                 120                 125

TTG AAA CGT AAG GAG CGG GAA CGT GCT CAA CAG TCC  AT GTAGGTGTTC      2710
Leu Lys Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser  Met
        130                 135                              140

TCCCTCTGTA GAGGAACGGC TGGACCCGCT CATCATTAAT TTTTTTTTTG TCTGTGAAG G  2770

TTT CCT GCG AGA CGC GGT AGC GCT GGC CCC AGT GGT ATC GCT CAA CTG     2818
Phe Pro Ala Arg Arg Gly Ser Ala Gly Pro Ser Gly Ile Ala Gln Leu
                145                 150                 155

CGC ATT TCC GAC CCG CCC GTT GCC ACC GGT AAC CCT CAG TCA ACC GAC     2866
Arg Ile Ser Asp Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp
            160                 165                 170

CTG ACC GCC GAC CCT ATG AAC CTC GAC GAT TTC ATC GTG CCC TTC GAA     2914
Leu Thr Ala Asp Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu
        175                 180                 185

TCT CCT TCG GAC CAC CCC TCG CCC AGT GCC GTC AAG ATT TCC GAC TCC     2962
Ser Pro Ser Asp His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser
    190                 195                 200
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Asp Ser Thr Pro Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln
225 230 235

CGG AAG AAC AGT GAA TTT GGC TAC GTC CCC CGT CGC GTG CGC AAG ACG 3106
Arg Lys Asn Ser Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr
240 245 250

AGT ATC GAC GAG CGT CAA TTT TTC TCA CTG CAG GTG CCG ACC CGA AAG 3154
Ser Ile Asp Glu Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys
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Val Asn His His Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp
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Thr Phe Gly Leu Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr
335 340 345

CAG TCG CAA TTC ACC TTC TCA CCC AGC GAG TCT CCG ATG GCC TCC GGT 3442
Gln Ser Gln Phe Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly
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CAT CCG TTT GCG AAC CTC TAT TCG CAT ACC CCG GTG GCT TCG TCC CTC 3490
His Pro Phe Ala Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu
365 370 375 380

AAC TCG ACG GAT TTC TTC TCT CCA CCG CCA TCA GGC TAC CAG TCC ACG 3538
Asn Ser Thr Asp Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr
385 390 395

GCA TCC ACG CCG CAG CCC ACC TAC GAC GGG GAC CAT TCC GTT TAT TTC 3586
Ala Ser Thr Pro Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe
400 405 410

GAT ATG CCG TCG GGC GAC GCG CGC ACC CAG CGC CGC ATT CCG AAC TAT 3634
Asp Met Pro Ser Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr
415 420 425

ATT TCG CAT CGG TCC AAC TTG TCT GCT TCG CTG CAG CCT CGG TAT ATG 3682
Ile Ser His Arg Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met
430 435 440

TTC AAC CAG AAC AAC CAT GAA CAG GCC AGT TCG TCG ACG GTG CAT TCG 3730
Phe Asn Gln Asn Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser
445 450 455 460

CCG AGC TAC CCC ATT CCC CAG CCG CAA CAT GTG GAC CCC ACT CAG GTG 3778
Pro Ser Tyr Pro Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val
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TTG AAC GCC ACC AAT TAC TCG ACC GGC AAC TCC CAC CAT ACC GGC GCC   3826
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ATG TTT TCA TTT GGA GCC GAT TCA GAT AAC GAG GAT GAC GAT GGT CAT   3874
Met Phe Ser Phe Gly Ala Asp Ser Asp Asn Glu Asp Asp Asp Gly His
        495                 500                 505

CAG CTG TCC GAG CGG GCT GGT CTG GCG ATG CCG ACT GAA TAT GGG GAC   3922
Gln Leu Ser Glu Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp
    510                 515                 520

GAG GAC GGG TTC TCG TCG GGC ATG CAG TGG GAT GGG CAG TTC CCG GGC   3970
Glu Asp Gly Phe Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly
525                 530                 535                 540

TCC TTC CAT TCG CTG CCG GGC TTT GGC CCT CAA CAT CGC AAG CAT GTT   4018
Ser Phe His Ser Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val
                545                 550                 555

ACC ATC GGG TCC ACG GAC ATG ATG GAC ACC CCC GAG GAG TGG AAT CAC   4066
Thr Ile Gly Ser Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His
            560                 565                 570

GGT GGC AGT TTG GGT CGG ACT CAT GGG TCG GTG GCT TCG GTC AGT GAG   4114
Gly Gly Ser Leu Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu
        575                 580                 585

GTG CGC AAC CGA GAG CAG GAC CCT CGC CGG CAG AAG ATT GCC CGC ACC   4162
Val Arg Asn Arg Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr
    590                 595                 600

ACG TCC ACC CCC AAT ACG GCC CAG CTG TTG CGC CAA AGC ATG CAC TCT   4210
Thr Ser Thr Pro Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser
605                 610                 615                 620

AAT AAC AAT ACG TCT CAT ACC TCC CCT AAT ACG CCG CCC GAG TCC GCC   4258
Asn Asn Asn Thr Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala
                625                 630                 635

CTG AGC AGC GCA GTT CCG TCC CGC CCG GCC AGT CCC GGG GGC AGC AAG   4306
Leu Ser Ser Ala Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys
            640                 645                 650

AAC GGC GAC CAA GGC AGC AAC GGA CCG ACC ACC TGC ACG AAC TGC TTC   4354
Asn Gly Asp Gln Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe
        655                 660                 665

ACT CAA ACC ACT CCG CTG TGG CGT CGG AAC CCA GAG GGC CAG CCA CTG   4402
Thr Gln Thr Thr Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu
    670                 675                 680

TGC AAT GCC TGC GGG TTG TTT TTG AAA TTG CAC GGT GTC GTG CGC CCT   4450
Cys Asn Ala Cys Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro
685                 690                 695                 700

CTG TCC CTG AAA ACG GAC GTT ATC AAA AAG CGC AAC CGT AGC AGT GCC   4498
Leu Ser Leu Lys Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala
                705                 710                 715

AAC AGC TTG GCG GTT GGG ACC TCC CGT GCG TCG AAG AAG ACA GCC CGC   4546
Asn Ser Leu Ala Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg
            720                 725                 730

AAG AAC TCG GTG CAG CAA GCA TCC GTC ACG ACT CCG ACA TCA AGC CGC   4594
Lys Asn Ser Val Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg
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GCT CAG AAT GGG ACT TCC TTC GAA TCC CCG CCC GCC GGC TTT AGT GCT    4642
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    750                 755                     760

GCC GCG GGA CGG TCG AAT GGG GTG GTA CCC ATT GCC GCC GCT CCT CCG    4690
Ala Ala Gly Arg Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro
765                     770                 775                 780

AAG GCA GCT CCC TCC GCA GCC GCC TCC CCT AGC ACG GGC CAG ACC CGC    4738
Lys Ala Ala Pro Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg
                785                 790                 795

AAC CCG ATC CAG GCT GCC CCG AAA CGT CAA CGA CGG CTG GAA AAG GCC    4786
Asn Pro Ile Gln Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala
            800                 805                 810

ACG GAG ATG GAA ACG GAC GAG GCT AAC AAG TCC GCG GGA GGC CGA TCC    4834
Thr Glu Met Glu Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser
        815                 820                 825

AAG GTG GTG CCT CTG GCA CCC GCC ATG CCA CCG GCA GCA GCC AAT CCG    4882
Lys Val Val Pro Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro
    830                     835                 840

GCG AAC CAT AGT ATT GCC GGA GGC CAA GGG GCT AGT CAG GAA TGG GAG    4930
Ala Asn His Ser Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu
845                     850                 855                 860

TGG TTG ACG ATG AGT CTGTAATGGC CGCGCTTACC TCTCTACTTC TCTACACTCG    4985
Trp Leu Thr Met Ser Leu
                865

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GTCTGAGCCT GTATTCATTG AAGTGTAGCC AGCTGTCGAA TGAGCTTTTT AACGATATTG    5585
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```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 866 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ser Gly Leu Thr Leu Gly Arg Gly Pro Gly Gly Val Arg Pro Thr
1               5                   10                  15

Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp Arg
            20                  25                  30

Ser Ser Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp Phe
        35                  40                  45

Ser Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp Gly
    50                  55                  60

Leu Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro Arg
65                  70                  75                  80

Pro Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu Ala
                85                  90                  95

Thr Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro Asn
            100                 105                 110

Gln Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser Leu
        115                 120                 125

Lys Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser Met Phe Pro Ala Arg
    130                 135                 140

Arg Gly Ser Ala Gly Pro Ser Gly Ile Ala Gln Leu Arg Ile Ser Asp
145                 150                 155                 160

Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp Leu Thr Ala Asp
                165                 170                 175

Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu Ser Pro Ser Asp
            180                 185                 190

His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser Thr Ala Ser Ala
        195                 200                 205

Ala Ile Pro Ile Lys Ser Arg Lys Asp Gln Leu Arg Asp Ser Thr Pro
    210                 215                 220

Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln Arg Lys Asn Ser
225                 230                 235                 240

Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr Ser Ile Asp Glu
                245                 250                 255

Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys Arg Pro Ala Glu
            260                 265                 270
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Ser Ser Pro Gln Val Pro Pro Val Ser Asn Ser Met Leu Ala His Asp
275 280 285

Pro Asp Leu Ala Ser Gly Val Pro Asp Tyr Ala Leu Asp Ala Pro Ser
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Ser Ala Phe Gly Phe His Gln Gly Asn His His Pro Val Asn His His
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Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp Thr Phe Gly Leu
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Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr Gln Ser Gln Phe
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Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly His Pro Phe Ala
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Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu Asn Ser Thr Asp
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Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr Ala Ser Thr Pro
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Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe Asp Met Pro Ser
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Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr Ile Ser His Arg
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Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met Phe Asn Gln Asn
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Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser Pro Ser Tyr Pro
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Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val Leu Asn Ala Thr
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Asn Tyr Ser Thr Gly Asn Ser His His Thr Gly Ala Met Phe Ser Phe
485 490 495

Gly Ala Asp Ser Asp Asn Glu Asp Asp Asp Gly His Gln Leu Ser Glu
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Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp Glu Asp Gly Phe
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Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly Ser Phe His Ser
530 535 540

Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val Thr Ile Gly Ser
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Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His Gly Gly Ser Leu
565 570 575

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Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu Val Arg Asn Arg
            580                 585                 590

Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr Thr Ser Thr Pro
        595                 600                 605

Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser Asn Asn Asn Thr
    610                 615                 620

Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala Leu Ser Ser Ala
625                 630                 635                 640

Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys Asn Gly Asp Gln
                645                 650                 655

Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe Thr Gln Thr Thr
            660                 665                 670

Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu Cys Asn Ala Cys
        675                 680                 685

Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro Leu Ser Leu Lys
    690                 695                 700

Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala Asn Ser Leu Ala
705                 710                 715                 720

Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg Lys Asn Ser Val
                725                 730                 735

Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg Ala Gln Asn Gly
            740                 745                 750

Thr Ser Phe Glu Ser Pro Pro Ala Gly Phe Ser Ala Ala Ala Gly Arg
        755                 760                 765

Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro Lys Ala Ala Pro
    770                 775                 780

Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg Asn Pro Ile Gln
785                 790                 795                 800

Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala Thr Glu Met Glu
                805                 810                 815

Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser Lys Val Val Pro
            820                 825                 830

Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro Ala Asn His Ser
        835                 840                 845

Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu Trp Leu Thr Met
    850                 855                 860

Ser Leu
865
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PATENT CLAIMS

1. A fungus, wherein the *areA* gene by recombinant DNA technology has been modified in a way by which it cannot be expressed in a way providing for a functional AreA activator.

2. The fungus of claim 1, wherein said inactivation has been obtained by deletion of all or parts the areA gene.

3. The fungus of claim 1, wherein said inactivation has been obtained by interfering with the regulation of the expression signals regulating the expression of the *areA* gene itself.

4. The fungus of claim 1, wherein said inactivation has been obtained by using anti-sense technology.

5. The fungus of claim 1, wherein said inactivation has been obtained by inserting extra DNA internally in the areA gene.

6. The fungus of any of claims 1 to 5, being a filamentous fungus, preferably belonging to a genus selected from the group comprising *Aspergillus*, *Trichoderma*, *Humicola*, *Candida*, *Acremonium*, *Fusarium*, and *Penicillium*

7. The fungus of claim 6, which belongs to a species selected from the group comprising *A. oryzae*, *A. niger*, *A. awamori*, *A. phoenicis*, *A. japonicus*, *A. foetidus*, *A. nidulans*, *T. reesei*, *T. harzianum*, *H. insulens*, *H. lanuginosa*, *F. graminearum*, *F. solani*, *P. chrysogenum*, and others.

8. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by deletion of the AreA gene, which method comprises

i) cloning of the *areA* gene from a fungus of interest,

ii) producing a DNA construct comprising the *areA* gene wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the construct, and

iv) selecting transformants which are *areA*$^-$.

9. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by using anti-sense technology, which method comprises

i) construction of an expression plasmid which gives rise to synthesis of an RNA molecule complementary to the mRNA transcribed from the areA gene,

ii) transformation of the host fungus with said expression plasmid and a suitable marker, either on separate plasmids or on the same plasmid,

iii) selection of transformants using said marker, and

iv) screening selected transformants for strains exhibiting a reduction in the synthesis of the AreA product.

10. A process for the production of a desired gene product, whereby a fungus according to any of the claims 1 to 7 is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

11. A process for the production of a desired gene product, whereby a fungus according to any of the claims 1 to 7, which has been transformed to integrate a DNA sequence coding for the desired gene product into the genome of the fungus in a functional manner, cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

12. A process for producing a desired polypeptide comprising cultivating a fungus in an appropriate growth medium and recovering said polypeptide from said culture, said fungus carrying a recombinant DNA construct capable of causing expression of said polypeptide or a precursor thereof in said fungus, said fungus further being characterized by producing lower amounts of functional AreA than the wild-type of said fungus.

13. A method according to claim 12, wherein said fungus has been modified to produce lower than wild-type amounts of AreA by a process comprising transforming a parent of said fungus with a DNA construct capable of causing reduced production of functional AreA when integrated in the geneome of said fungus.

14. A method according to claim 12, wherein said polypeptide is secreted to the extracellular medium by said fungus.

15. A method according to claim 12, wherein said fungus produces higher amounts of said polypeptide than a similar fungus where said similar fungus produces AreA in amounts similar to those produced by the wild-type of said fungus, said similar fungus being identical to said fungus in all other respects.

16. The process of claim 10 or 11 to 15, wherein said gene product is a secreted protein.

17. The process of any of the claims 10 to 16, wherein said desired gene product is an industrial peptide or protein, preferably an enzyme.

18. The process of claim 17, wherein said enzyme is selected from the group comprising a protease, lipase, cutinase, cellulase, chymosin.

19. The process of any of the claims 10 to 116, wherein said desired gene product is a therapeutically active peptide or protein.

20. The process of claim 19, wherein said therapeutically active peptide or protein is selected from the group comprising insulin, growth hormone, glucagon, somatostatin, interferon, PDGF, factor VII, factor VIII, urokinase, tPA, EPO, or TPO.

21. A gene product produced in accordance with any of the processes 10 to 20.

22. A DNA sequence coding for the *areA* gene from *A. oryzae* (SEQ ID No. 1) or functional alleles thereof.

23. An AreA activator from *A. oryzae* (SEQ ID No. 2).

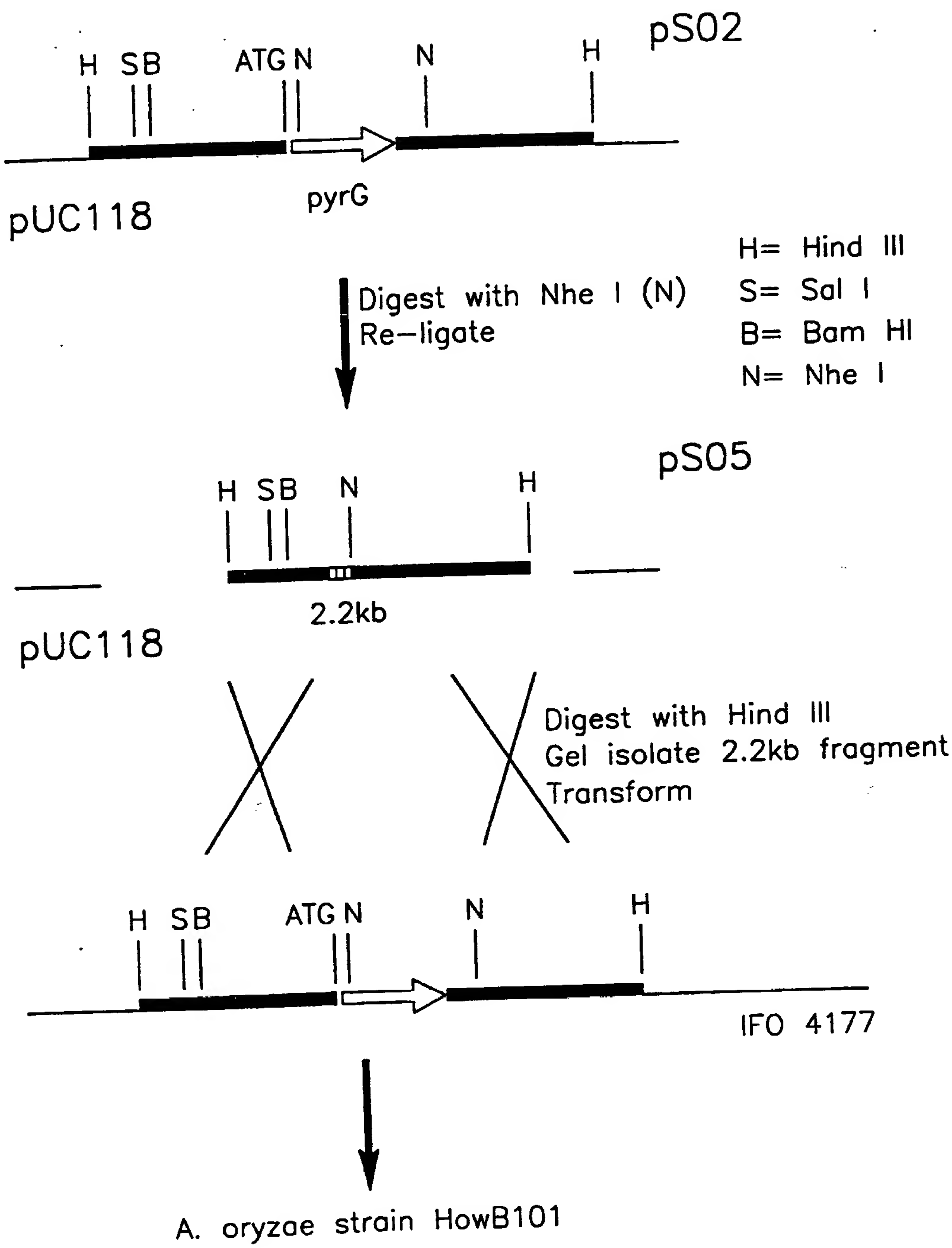
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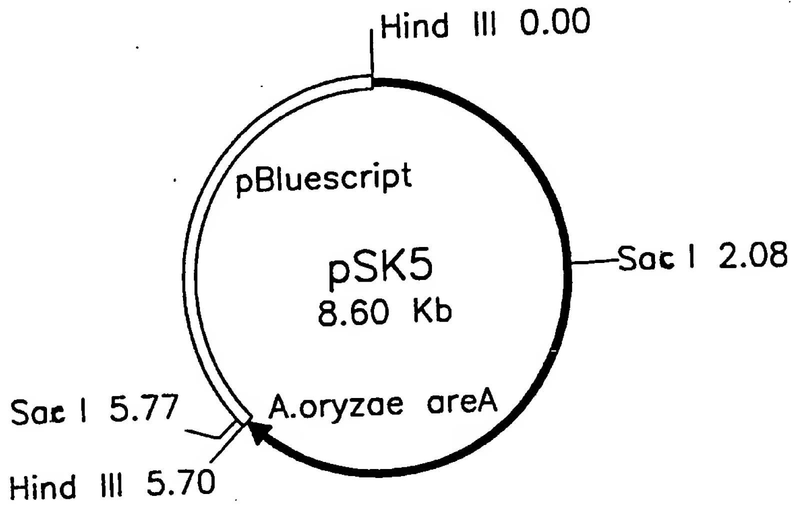
Fig. 1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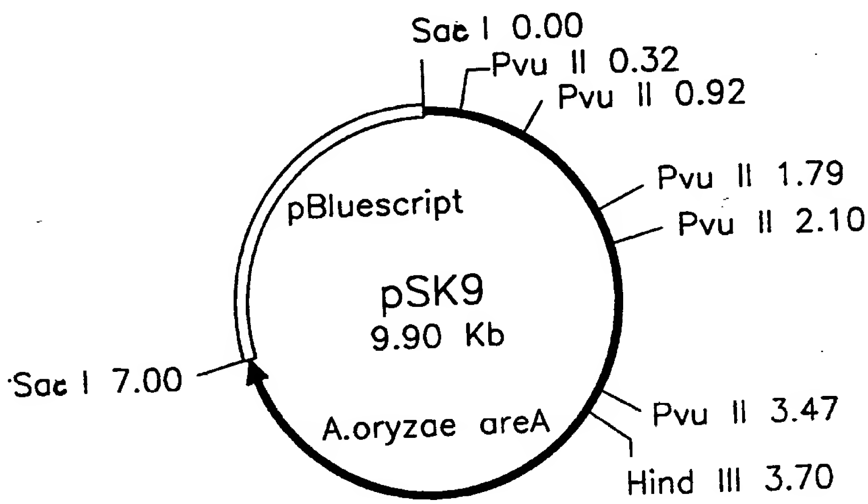
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Pvu II 0.32
Pvu II 0.92
Pvu II 1.79
Pvu II 2.10
pBluescript
pSK9
9.90 Kb
Sac I 7.00
A.oryzae areA
Pvu II 3.47
Hind III 3.70

Fig. 2

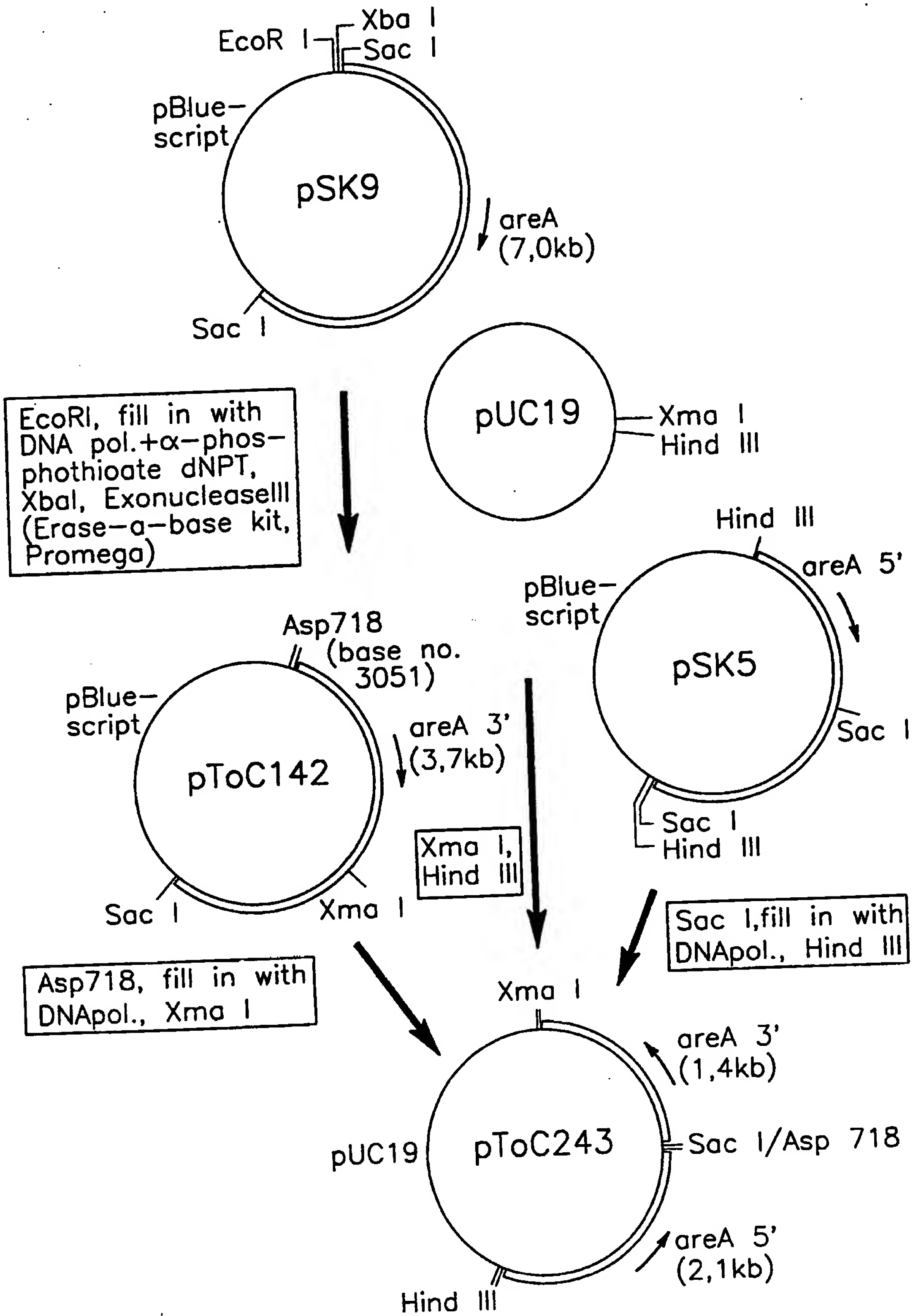
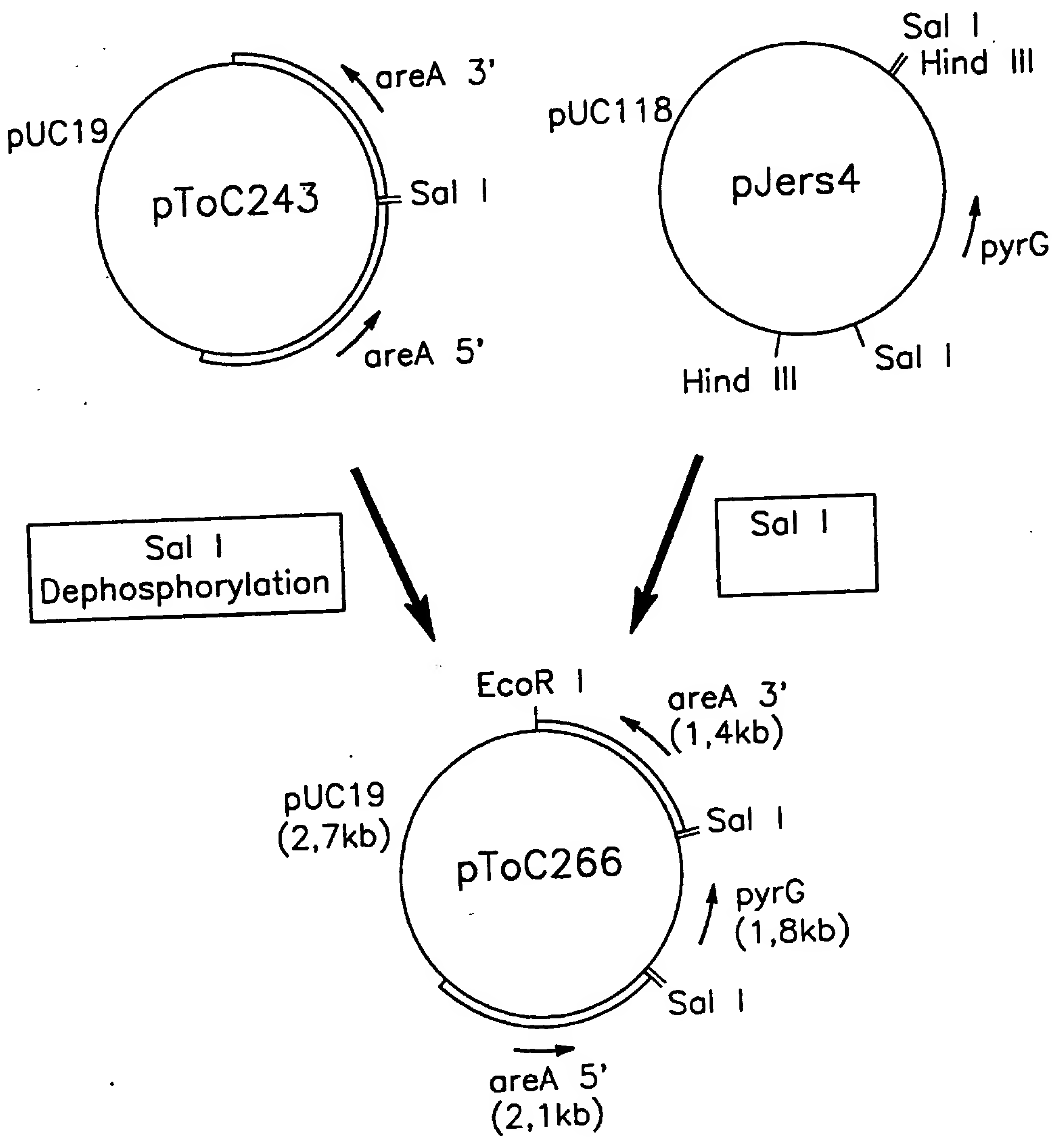
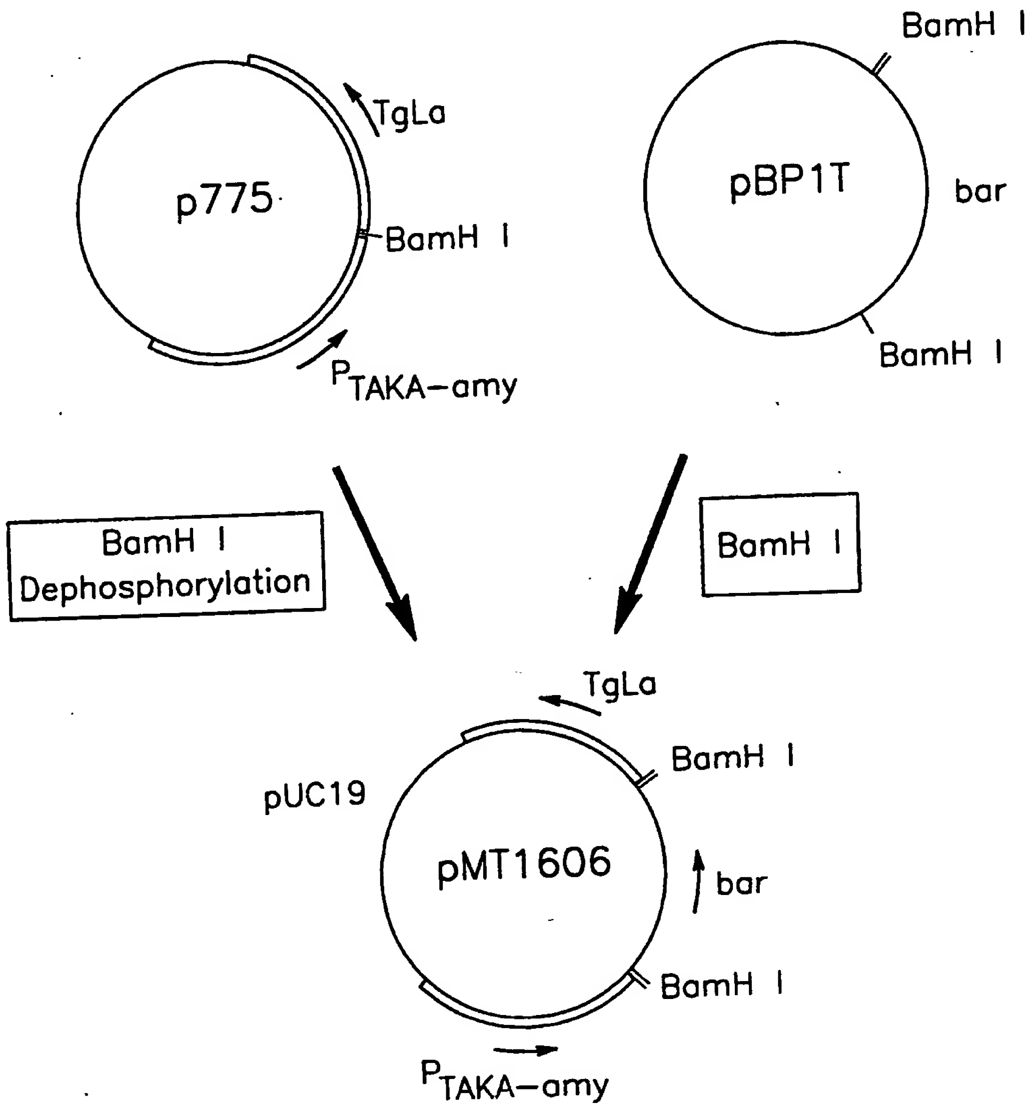

Xba I
EcoR I
Sac I
pBlue-
script
pSK9
areA
(7,0kb)
Sac I
pUC19
Xma I
Hind III
EcoRI, fill in with DNA pol.+α-phosphothioate dNPT, XbaI, ExonucleaseIII (Erase-a-base kit, Promega)
Hind III
areA 5'
pBlue-
script
pSK5
Sac I
Sac I
Hind III
Asp718
(base no. 3051)
pBlue-
script
pToC142
areA 3'
(3,7kb)
Sac I
Xma I
Xma I,
Hind III
Sac I,fill in with DNApol., Hind III
Asp718, fill in with DNApol., Xma I
Xma I
areA 3'
(1,4kb)
pUC19
pToC243
Sac I/Asp 718
areA 5'
(2,1kb)
Hind III

Fig. 3a



pUC19
pToC243
areA 3'
Sal I
areA 5'
pUC118
pJers4
Sal I
Hind III
pyrG
Hind III
Sal I
Sal I
Dephosphorylation
Sal I
EcoR I
areA 3'
(1,4kb)
pUC19
(2,7kb)
pToC266
Sal I
pyrG
(1,8kb)
Sal I
areA 5'
(2,1kb)

Fig. 3b



p775
TgLa
BamH I
PTAKA-amy
pBP1T
BamH I
bar
BamH I
BamH I
Dephosphorylation
BamH I
pMT1606
TgLa
BamH I
pUC19
bar
BamH I
PTAKA-amy

Fig. 4

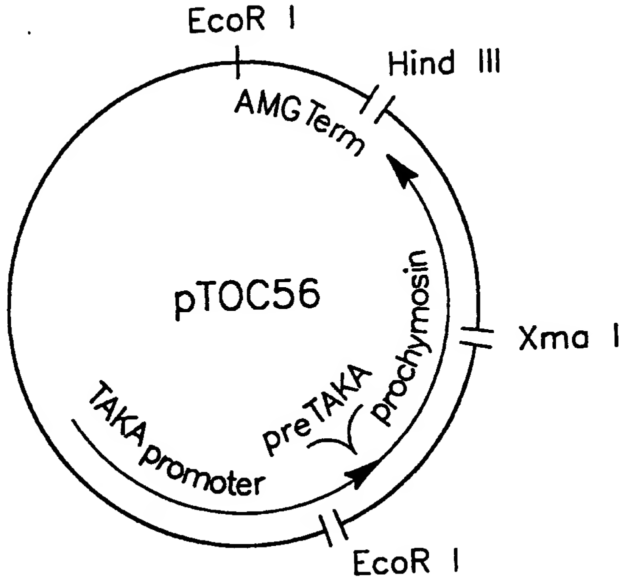

EcoR I
Hind III
AMG Term
pTOC56
prochymosin
Xma I
preTAKA
TAKA promoter
EcoR I

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00254

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/80, C12N 1/15, C07K 14/38, C07H 21/04 // C12N 15/67

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, CLAIMS, EMBL, GENESEQ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molec. gen. Genet., Volume 126, 1973, HERBERT N. ARST ET AL, "Nitrogen Metabolite Repression in Aspergillus nidulans", page 111 - page 141, see page 117 and table 4 --	1-21
Y	WO 9217595 A1 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.), 15 October 1992 (15.10.92), page 4, line 11 - line 28 --	1-21

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 November 1995	14-11- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00254

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Molecular Microbiology, Volume 7, No 1, 1993, M. Stankovich et al, "C-terminal truncation of the transcriptional activator encoded by areA in Aspergillus nidulans results in both loss-of-function and gain-of-function phenotypes" page 81 - page 87 --	22-23
X	Gene, Volume 95, 1990, Mark X. Caddick et al, "Nitrogen regulation in Aspergillus: are two fingers better than one?" page 123 - page 127 --	22-23
X	The EMBO Journal, Volume 9, No 5, 1990, Bernard Kudla et al, "The regulatory gene areA mediating nitrogen metabolite repression in Aspergillus nidulans. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger" page 1355 - page 1364 --	22-23
A	The EMBO Journal, Volume 5, No 5, 1986, MARK X. CADDICK ET AL, "Cloning of the regulatory gene areA mediating nitrogen metabolite repression in Aspergillus nidulans" page 1087 - page 1090 --	1-21
A	US 5179003 A (DIETER H. WOLF ET AL), 12 January 1993 (12.01.93), column 2, line 16 - line 42 --	1-21
A	EP 0206783 A2 (THE SALK INSTITUTE BIOTECHNOLOGY INDUSTRIAL ASSOCIATES, INC.), 30 December 1986 (30.12.86), page 5, line 3 - line 15; page 8, line 13 - line 17, abstract -- --------	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK95/00254

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

According to rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features" i.e. features that define a contribution which each of the inventions makes over prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying novel technical feature.

Accordingly the following inventions were found:

I Claims 1-21 directed to a fungus wherein the areA gene has been modified so that the fungus does not produce proteases

II Claims 22-23 directed to an areA gene from Aspergillus oryzae

The two groups do not form a general inventive concept.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/10/95

International application No.
PCT/DK 95/00254

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9217595	15/10/92	AU-B- 661844	10/08/95
		AU-A- 1750592	02/11/92
		EP-A- 0578746	19/01/94
		JP-T- 6506117	14/07/94
		US-A- 5324660	28/06/94
US-A- 5179003	12/01/93	DE-A- 3804890	13/07/89
		EP-A- 0327797	16/08/89
		JP-A- 2002385	08/01/90
EP-A2- 0206783	30/12/86	AU-A- 5912586	24/12/86
		JP-A- 62036183	17/02/87